Deterministic Pressure-Induced Dissociation of Vicilin, the 7S Storage Globulin from Pea Seeds: Effects of pH and Cosolvents on Oligomer Stability†

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ABSTRACT: A thermodynamic characterization of subunit association of vicilin, a storage protein from pea seeds, was performed using a combination of hydrostatic pressure and fluorescence spectroscopy. Application of pressure up to 2.4 kbar caused dissociation of vicilin subunits, as revealed by (1) size-exclusion FPLC of pressurized samples, (2) fluorescence anisotropy measurements of a dansyl–vicilin conjugate under pressure, and (3) quenching of the intrinsic fluorescence of vicilin. Pressure dissociation data were well described by a model for dissociation of a trimer. This enabled calculation of the standard molar volume change of association (ΔV°) and the equilibrium dissociation constant at atmospheric pressure (K_o); at pH 10 these were found to be $\Delta V^\circ = 146$ mL/mol and $K_o = 2.2 \times 10^{-15}$ M^2, respectively, corresponding to $C_{1/2}$ (the concentration of protein at 50% dissociation at atmospheric pressure) = 18 nM and a stabilization free energy of −19.6 kcal/mol for the oligomer. Vicilin exhibited an anomalously low dependence on protein concentration for pressure dissociation. This appeared related to conformational changes in the dissociated subunits, which caused a loss of ca. 5 kcal/mol in the free energy of association and to structural/energetic heterogeneity in the population of oligomers. Pressure dissociation was markedly pH-dependent, with a stabilization free energy loss of 3.4 kcal/mol upon raising pH from 9 to 10. Circular dichroism and intrinsic fluorescence lifetime measurements at atmospheric pressure showed that the structure of vicilin was largely unaffected by pH in the range investigated. These results suggest that the effect of pH may involve deprotonation of lysine residues participating in salt bridges between vicilin subunits. Pressure dissociation of vicilin was significantly inhibited by addition of salts (NaCl, KCl, LiCl) or glycerol. Dissociation curves obtained in the presence of salts enabled calculation of the free energies of stabilization (ranging from ca. −1.2 to −2.4 kcal/mol) of the vicilin oligomers by these cosolvents. The similar effects of salts or glycerol suggest a common mechanism of stabilization of the oligomer involving exclusion of the cosolvents from the protein interface and preferential hydration of the protein.

Vicilin, the 7S storage protein of peas (*Pisum sativum*), corresponds to up to 35% of the total protein content of the seeds and consists of three major subunits of 50 kDa assembled to form a 150-kDa oligomer (Gatehouse et al., 1984; Bewley & Black, 1985). Some of the 50-kDa subunits are nicked by posttranslational proteolysis soon after biosynthesis (Gatehouse et al., 1982, 1983; Lycett et al., 1983), and the resulting peptides (ranging from 12.5 to 33 kDa) remain associated with the intact subunits in the native 150-kDa oligomer (Gatehouse et al., 1982).

Considerable interest has been focused on the relationships between structure and physicochemical properties of seed proteins used in the food and biotechnology industries (Kinsella et al., 1979; Wright & Bumstead, 1984; Wright, 1985; Caer et al., 1990; Chambers et al., 1990; Baniel et al., 1992). Investigation of the oligomeric structure of seed storage globulins should provide interesting information on subunit interactions which directly influence their useful physicochemical and functional (e.g., gelling or emulsification) properties. In addition, an understanding of protein–protein interactions is also relevant to genetic engineering studies of seed globulins which are currently hampered by incorrect folding and reassociation of the recombinant subunits (Watson et al., 1988). *In vivo,* the correct association and assembly of oligomers is also important for efficient packing of storage proteins into the protein bodies of the seeds. The stability of the oligomeric structure of seed storage globulins has been investigated by changes in chemical composition of the medium, such as in extremes of pH (Gueguen et al., 1988), presence of denaturants or detergents (Brand & Johnson, 1956; Grant & Lawrence, 1964; Chandra et al., 1985; Guerrieri & Cerletti, 1990), or addition of chaotropic salts (Chambers et al., 1990). To our knowledge, no studies have been reported on the dissociation of seed storage globulins by use of a physical method to perturb subunit association under conditions not directly affecting the secondary or tertiary structures of the protein. This would enable a more direct assessment of effects on the quaternary structure of the aggregates.

In this study, protein–protein and protein–solvent interactions of vicilin were investigated by using hydrostatic pressure as an external variable to perturb the equilibrium of subunit association. The combination of high-pressure techniques and optical spectroscopy has been successfully used to investigate the dissociation of several biological polymers [for reviews, see Heremans (1982), Weber and Drickamer (1983), Weber (1987), and Silva and Weber (1993)], including such complex aggregates as brome mosaic virus (Silva & Weber, 1988), the extracelular giant hemoglobin from *Glossoscolex paulistus* (Silva et al., 1989; Bonafe et al., 1991), mitochondrial ATP synthase (Dreyfus et al., 1988), and F-actin (Garcia et al., 1992). Pressure-induced dissociation of vicilin was monitored by measurements of the protein intrinsic fluores-

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cence and the fluorescence anisotropy of a dansyl-labeled vicilin conjugate. The extent of dissociation was also determined by FPLC[1] gel-permeation chromatography of pressure-treated vicilin samples. After decompression, intermediate states of assembly of vicilin could be detected, suggesting that the dissociated subunits undergo conformational changes that lead to loss of association free energy and incomplete reassembly of the oligomer. Pressure–dissociation curves enabled calculation of the standard molar volume change of association of vicilin, as well as the free energies of stabilization of the oligomer in different conditions. Increasing pH from neutrality to pH 10 led to a marked increase in the degree of pressure dissociation of vicilin, whereas presence of cosolvents such as salts or glycerol produced a marked stabilization of the oligomer. These results are interpreted in terms of the nature of the forces responsible for the assembly of subunits in the vicilin oligomer.

MATERIALS AND METHODS

Purification of Vicilin. Vicilin was isolated from pea seeds and purified essentially as described by Lambert et al. (1986), with an extra step of precipitation at 45% ammonium sulfate prior to isolation of the 75–99% ammonium sulfate pellet. Throughout the purification, solutions contained a cocktail of protease inhibitors containing 0.5 μg/mL leupeptin, 0.07 μg/mL pepstatin, 2 μg/mL PMSF, and 0.05 μg/mL soybean trypsin inhibitor. Vicilin stock solutions (4–7 mg/mL) were stored at 4 °C in 50 mM ammonium bicarbonate, pH 8.0, and were stable for 1–2 months. Under nondenaturing conditions (absence of SDS), polyacrylamide gel electrophoresis of vicilin samples revealed a single band stained with Coomassie Blue (not shown). The electrophoretic pattern was unchanged in the presence of β-mercaptoethanol. Size-exclusion FPLC of native vicilin revealed a single peak of 150 kDa (see Results). SDS–PAGE of purified vicilin revealed the presence of a major 50-kDa band (~60% of the total protein) plus seven post-translational proteolytic fragments (Gatehouse et al., 1984) ranging from 12.5 to 33 kDa. All seven peptide fragments were shown to be immunoreactive with an anti-vicilin antiserum obtained by immunizing rabbits with the 50-kDa subunit excised from SDS–PAGE gels (not shown). Protein concentration was determined according to Lowry et al. (1951).

Steady-State Fluorescence Measurements. Fluorescence emission spectra were measured at 23 °C on an ISS (Champaign, IL) GREG 200 spectrofluorometer interfaced to an IBM personal computer. Excitation was at 272 nm (which corresponded to the peak in the vicilin excitation spectrum), and a bandpass of 8 nm was used for both excitation and emission. Unless otherwise indicated, vicilin concentration was 3.3 μM (assuming a molecular mass of 150 kDa for oligomeric vicilin). Fluorescence measurements under pressure were performed using the pressure cell equipped with quartz optical windows described by Paladini and Weber (1981). The experiments shown are representative of at least four experiments performed with different vicilin preparations which displayed slightly different sensitivities to pressure dissociation. Unless otherwise indicated, pressure dissociation was carried out in standard medium containing 50 mM ammonium bicarbonate, pH 10. Control experiments at pH 8–10 utilizing Tris-HCl instead of bicarbonate yielded essentially the same results.

[1] Abbreviations: CD, circular dichroism; 1,5-dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

For fluorescence anisotropy measurements, vicilin was covalently labeled with 1,5-dansyl chloride (Molecular Probes, Eugene, OR). A solution of vicilin (4.2 mg/mL in a total volume of 1 mL) in 50 mM ammonium bicarbonate buffer (pH 8.0) was filtered through a Nalgene cellulose acetate filter (0.2 μm pore size), and 6.3 μL of a freshly prepared stock solution of 1,5-dansyl chloride in dimethylformamide (3.5 mg/mL) was added. Reaction was carried out at room temperature in the dark, with occasional gentle stirring. After 60 min, the sample was dialyzed for 48 h against 50 mM Tris-HCl buffer (pH 10), with four changes of the dialysis buffer. Samples of the dansyl–vicilin conjugate were analyzed by SDS–PAGE, which showed that dansyl labeling (visualized by UV illumination of the unstained gel) appeared to be uniformly distributed between the intact 50-kDa vicilin subunit and the lower molecular weight polypeptides (data not shown). Fluorescence anisotropy of dansyl–vicilin was measured with 8-nm bandpass with excitation at 340 nm and emission at 500 nm. A Corning 7-54 filter was used in the excitation, and a KV-470 was used in the emission. Anisotropy values measured in the high-pressure bomb were corrected for birefringence of the quartz windows, as described by Paladini and Weber (1981).

Determination of Association Volume and Equilibrium Constant. The thermodynamic equilibrium between the aggregate and N elementary subunits of an oligomeric protein is described by the relation (Erijman & Weber, 1991; Weber, 1992)

$$K = N^N \alpha^N C^{N-1}/(1-\alpha) \quad (1)$$

where K is the dissociation constant, α is the degree of dissociation of the aggregate, and C is the molar concentration of protein (expressed as aggregate). According to Le Chatelier's principle, application of hydrostatic pressure to this system will result in a change in the apparent dissociation constant of the aggregate–subunit equilibrium. The effect of hydrostatic pressure (p) on the apparent dissociation constant ($K_{(p)}$) of such system can be described by (Paladini & Weber, 1981; Weber, 1987; Erijman & Weber, 1991)

$$K_{(p)} = K_o \exp(p\Delta V^o/RT) \quad (2)$$

where K_o is the dissociation constant at atmospheric pressure, ΔV^o is the standard molar volume change upon association of the subunits to form the aggregate, and R and T have their usual meanings. From eqs 1 and 2, the degree of dissociation of the oligomer as a function of pressure is given by

$$\alpha^N_{(p)}/(1-\alpha_{(p)}) = (K_o/N^N C^{N-1}) \exp(p\Delta V^o/RT) \quad (3)$$

The degree of dissociation of oligomeric proteins upon application of pressure can be conveniently followed by a number of spectroscopic techniques, including fluorescence emission [for reviews, see Weber (1987) and Silva and Weber (1993)]. In the present work, quenching of the fluorescence of vicilin samples upon application of pressure was utilized to monitor dissociation of the aggregate. Native oligomeric vicilin consists of a structural trimer (Gatehouse et al., 1984). Accordingly, our data on the changes in vicilin fluorescence upon application of pressure were analyzed with eq 3 by making $N = 3$. For calculation of the degree of dissociation at each pressure, determination of the maximal fluorescence change (ΔF_{max}) at complete dissociation was required. This was achieved by performing iterative linear fits of $\ln(\alpha^3/1-\alpha)$ versus pressure, according to eq 3. Degrees of dissociation were then obtained from the relation

$$\alpha_{(p)} = \Delta F/\Delta F_{max} \quad (4)$$

The standard volume change of association was obtained from the slope of the linear plot of $\ln(\alpha^3/1-\alpha)$ versus pressure, while the equilibrium constant at atmospheric pressure was obtained from the intercept in this plot (see Figure 4). Alternatively, direct fits of the change in α as a function of pressure were performed according to eq 3, utilizing a computer program kindly provided by Dr. G. Weber (University of Illinois at Urbana–Champaign). Theoretical curves for pressure dissociation were thus generated and fit to the experimental data, as shown in Figures 5, 6, 8, 10, and 11.

Time-Resolved Fluorescence Measurements. The intrinsic fluorescence lifetimes of vicilin were measured on a multifrequency phase-modulation fluorometer at 12 different frequencies from 7 to 250 MHz. Excitation at 282 nm was provided by the harmonic content of a Coherent (Antares model) Nd:YAG mode-locked, cavity-dumped, frequency-doubled laser used to pump an externally frequency-doubled Rhodamine 6G dye laser (Coherent model 700). Excitation was at "magic angle" configuration, and emission was collected through a Hoya UV-30 filter. A reference sample of *p*-terphenyl in cyclohexane (lifetime = 1.05 ns) was used. Lifetime data were analyzed with the Globals Unlimited software (Beechem et al., 1991) in terms of sums of exponential decays or with continuous lifetime distributions. Standard deviations of ±0.2° and ±0.004 in phase and modulation measurements, respectively, were used for the calculation of reduced χ^2 for the fits. Comparison between different fluorescence decay models was performed by F-statistics analysis, as previously described (Ferreira & Verjovski-Almeida, 1989, 1991).

Circular Dichroism. Ultraviolet circular dichroism spectra of vicilin were measured at 23 °C on a Jobin-Yvon CD6 spectropolarimeter interfaced to a PC-AT microcomputer, using 0.1-cm path length quartz cells. UV-CD spectra shown are averages of three scans for each sample. Vicilin concentration in CD measurements was 2.3 μM.

Size-Exclusion Fast Protein Liquid Chromatography (FPLC). Size-exclusion chromatography was performed on a Pharmacia-LKB FPLC apparatus equipped with a LCC-500 controller, using a precalibrated Superose 12 HR 10/30 column (10 × 300 mm; exclusion limits 1–300 kDa). The column was equilibrated with buffer containing 30 mM Tris-HCl and 150 mM NaCl at different pH values as indicated under Results. Elution was monitored by absorption at 214 nm. The flow rate was 0.3 mL/min. Column calibration was done with a set of proteins of known molecular weights: thyroglobulin, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin, myoglobin, and cytochrome *c*.

RESULTS

Pressure-Induced Dissociation of Vicilin. Application of hydrostatic pressure up to 2.4 kbar produced significant changes in the fluorescence of vicilin. Figure 1 shows the effects of pressure on the intrinsic fluorescence of vicilin at different pH values. Vicilin displayed characteristic tyrosine fluorescence emission with maximal fluorescence intensity at 307 nm, compatible with the absence of tryptophan residues in its amino acid composition (Watson et al., 1988). At pH 7 (Figure 1A) application of 2.4 kbar for 1 h promoted approximately 20% quenching of vicilin fluorescence. Upon release of pressure, partial recovery of the fluorescence emission to about 90% of the initial intensity was observed (Figure 1A, dashed line). Alkalinization of the medium to pH 8, 9, or 10 (Figure 1, panels B–D) led to enhanced sensitivity of vicilin

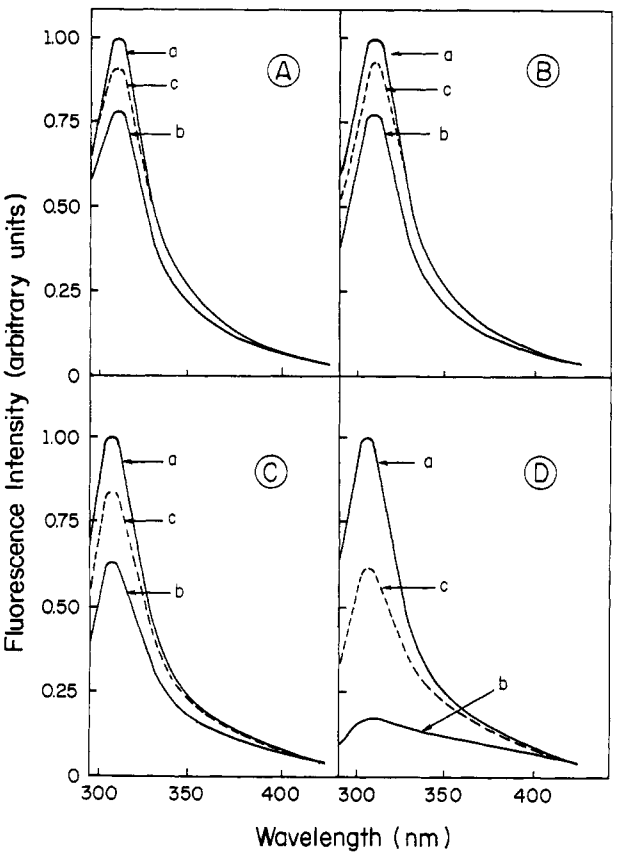


FIGURE 1: Pressure effects on the fluorescence emission of vicilin. Intrinsic fluorescence emission spectra of vicilin were measured at atmospheric pressure *before* pressure application (trace a), at 2.4 kbar for 1 h (trace b), and at atmospheric pressure *after* pressure release (trace c). Panels A–D show experiments performed at pH 7, 8, 9, or 10, respectively. Samples contained 3.3 μM vicilin in 50 mM ammonium bicarbonate buffer, at the indicated pH values.

fluorescence to pressure. At 2.4 kbar, vicilin fluorescence was quenched by 25%, 37%, or 80% at pH 8, 9, or 10, respectively. In addition, fluorescence intensity was recovered to a lesser extent after release of pressure at alkaline pH. No spectral shifts or changes in average emission energy of the fluorescence were observed upon application of pressure or at different pH values. At atmospheric pressure, alkalinization of the medium from pH 8 to 10 caused slight (10–15%) fluorescence quenching, and this was fully reversible upon return to pH 8 (not shown).

Pressures up to 3 kbar have been shown to exert minimal direct effect on the tertiary structure of proteins (Heremans, 1982; Weber & Drickamer, 1983; Weber, 1987). In this same pressure range, several protein aggregates have been shown to undergo subunit dissociation [for reviews, see Weber (1987) and Silva and Weber (1993)]. This suggested that the pressure-induced changes in vicilin fluorescence could be related to subunit dissociation. To examine this possibility, size-exclusion gel filtration FPLC analysis of vicilin samples which had been subjected to pressure was carried out. Figure 2 shows gel filtration elution profiles of control and pressurized vicilin samples at different pH values. From pH 8 to 10, control vicilin samples eluted as a single peak of 150 kDa (Figure 2, panels A, C, and E), indicating that the hydrodynamic volume of vicilin was not affected by changing pH in this range at atmospheric pressure. Pressurized vicilin samples were kept at 2.4 kbar for 1 h; pressure was then released, and aliquots were applied onto the column. Figure 2 (panels B, D, and F) shows elution profiles of samples pressurized at pH 8, 9, or 10, respectively. Application of

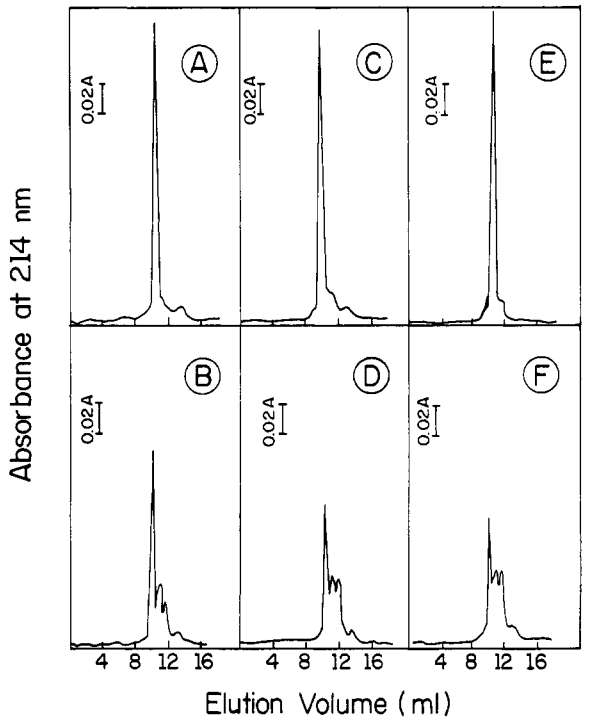


FIGURE 2: FPLC analysis of control and pressurized vicilin. Aliquots from vicilin samples at pH 8, 9, or 10 were subjected to size-exclusion FPLC analysis before application of pressure (panels A, C, and E, respectively). Samples were then pressurized at 2.4 kbar for 1 h at the indicated pH values, pressure was released, and aliquots were immediately applied onto the FPLC column and chromatographed (panels B, D, and F, at pH 8, 9, or 10, respectively). Total protein applied in each run was 6 μg. Chromatography conditions are described under Materials and Methods.

pressure led to accumulation of lower molecular weight peaks eluting at positions corresponding to approximately 50 and 75 kDa in the calibrated gel filtration column, probably corresponding to the intact 50-kDa subunit and to an intermediate state of assembly of the vicilin oligomer, respectively. Pressure dissociation of vicilin and accumulation of assembly intermediates increased at alkaline pH: at pH 8, 60% of the vicilin was found associated in the 150-kDa oligomer after pressure release, whereas at pH 9 or 10 the fraction of 150-kDa oligomer decreased to 50 or 45%, respectively (Figure 2B, D, and F). The elution profiles of pressurized samples did not change over a period of 24 h following pressure release (not shown), indicating that the 75-kDa component was a stable trapped intermediate of vicilin assembly.

Direct confirmation that vicilin fluorescence quenching reported pressure-dissociation of the oligomer was obtained from measurements of the fluorescence anisotropy of a dansyl–vicilin conjugate under pressure. Fluorescence anisotropy measurements yield direct information on the size of the rotating particle to which the fluorophore is attached and have been utilized to monitor the decrease in size upon pressure-dissociation of oligomeric proteins (Paladini & Weber, 1981; Thompson & Lakowicz, 1984; Coelho-Sampaio et al., 1991). The degree of dissociation of the oligomer can be calculated from fluorescence anisotropy measurements as (Paladini & Weber, 1981)

$$\alpha_{(p)} = [1 + Q(r_{(p)} - r_{(D)})/(r_{(A)} - r_{(p)})]^{-1} \qquad (6)$$

where Q is the ratio of the fluorescence quantum yields of the aggregate and dissociated forms, $r_{(p)}$ is the fluorescence anisotropy at a given pressure, and $r_{(A)}$ and $r_{(D)}$ are anisotropies

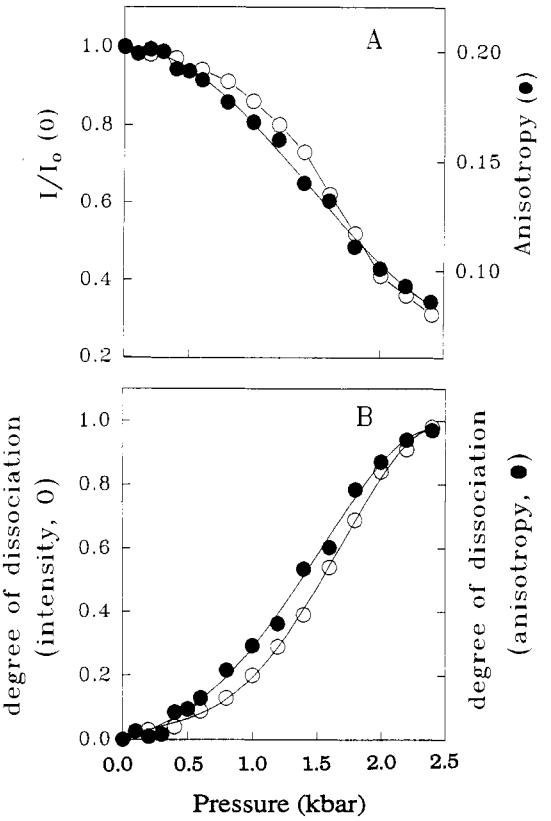


FIGURE 3: Correlation between fluorescence anisotropy and intensity changes as a function of pressure. (Panel A) Vicilin was covalently labeled with 1,5-dansyl as described under Materials and Methods, and fluorescence anisotropy measurements of the dansyl–vicilin conjugate were performed as a function of applied hydrostatic pressure (●). For comparison, the ratio I/I_o expressing intrinsic fluorescence intensity changes (λ_{em} = 305 nm) of unlabeled vicilin as a function of pressure is also shown (○). Samples contained 2.7 μM dansyl-labeled vicilin in 50 mM Tris-HCl, pH 10, or 3.3 μM unlabeled vicilin in 50 mM ammonium bicarbonate, pH 10. (Panel B) Degrees of dissociation of vicilin as a function of pressure were calculated as described under Materials and Methods from anisotropy data on dansyl–vicilin (●) or intrinsic fluorescence of unlabeled vicilin (○).

of the aggregate and fully dissociated protein, respectively. Figure 3A shows that both intrinsic fluorescence intensity of unlabeled vicilin and anisotropy of dansyl–vicilin decreased upon application of pressure. Figure 3B shows the degrees of dissociation of the oligomer as a function of pressure, calculated from intrinsic fluorescence quenching or anisotropy measurements according to eqs 4 or 6, respectively.

In the interpretation of the fluorescence quenching of vicilin under pressure it is also important to note that examination of the known amino acid sequence and the location of the sites of proteolytic cleavage of the protein (Lycett et al., 1983; Gatehouse et al., 1984) indicates that the tyrosine residues of vicilin are distributed along the intact subunit, so that all peptide fragments contain tyrosine residues. These considerations rule out the possibility that the fluorescence changes observed upon application of pressure might reflect primarily dissociation of the smaller fragments from the oligomer.

Thermodynamic Parameters of Vicilin Association. Pressure–dissociation data for vicilin were analyzed with a model for dissociation of a trimer, as described under Materials and Methods. The linear plot in Figure 4 shows that data obtained for pressure-dissociation of vicilin following fluorescence quenching (as shown in Figure 3) could be well described with this model. Furthermore, this analysis enabled calculation of the standard molar volume change of association of subunits and the equilibrium dissociation constant at atmospheric pressure. An association volume change (ΔV^o) of

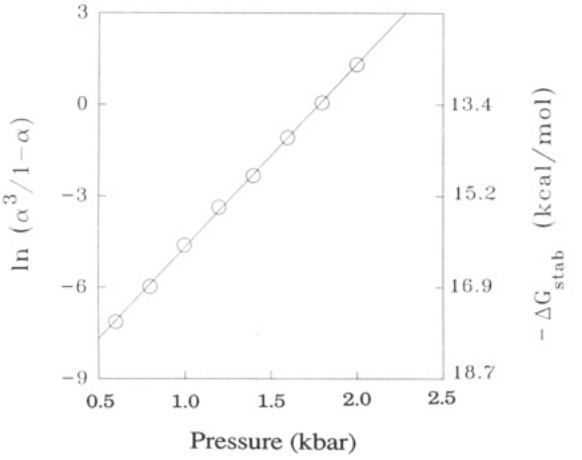


FIGURE 4: Linear plot of ln ($\alpha^3/1 - \alpha$) versus pressure. Pressure dissociation of vicilin was measured at pH 10 by following the decrease in fluorescence intensity at 305 nm, as shown in Figure 3. Degrees of dissociation at each pressure were calculated and analyzed with a model for dissociation of a trimer (see Materials and Methods for details). Stabilization free energies, ΔG_{stab}, were calculated from the relation $\Delta G_{stab} = RT \ln K_{diss}$.

146 mL/mol was calculated, with an equilibrium dissociation constant (K_0) of 2.2×10^{-15} M^2, corresponding to $C_{1/2} = 18$ nM and $\Delta G_{stab} = -19.6$ kcal/mol at 20 °C. Figure 4 also shows the decrease in ΔG_{stab} of the vicilin oligomer at increasing pressures.

Concentration Dependence for Pressure Dissociation. Pressure–dissociation studies of complex protein aggregates such as brome mosaic virus (Silva & Weber, 1988), erythrocruorin from the worm *G. paulistus* (Silva et al., 1989), mitochondrial ATP synthase (Dreyfus et al., 1988), and F-actin (Garcia et al., 1992) have shown that these large aggregates display small or negligible dependence on protein concentration of the pressure required to produce half-maximal dissociation. These findings are in contrast with the observed dependence on protein concentration for dissociation of dimers (Verjovski-Almeida et al., 1986; Ruan & Weber, 1988; Erijman & Weber, 1991; Coelho-Sampaio et al., 1991). For tetrameric glyceraldehyde-phosphate dehydrogenase an anomalously low dependence on protein concentration has also been reported (Ruan & Weber, 1989).

Figure 5 shows pressure–dissociation curves obtained at 2 μM (closed circles) or 16 μM (open circles) vicilin. The curves at these two protein concentrations were very similar, and a difference of about 200 bar was found in the pressure required for half-maximal dissociation. This experimental difference in pressure ($dp_{1/2}$) can be compared with the theoretical expected value, calculated for the dissociation of a trimer at the two concentrations tested, C_1 and C_2, as

$$dp_{1/2} = [(N - 1/N)RT \ln(C_2/C_1)]/(\Delta V^o/N) \qquad (7)$$

For the vicilin concentrations employed in Figure 5, a $dp_{1/2}$ value of 700 bar would be expected.

Hysteresis and Conformational Changes of Pressure-Dissociated Vicilin Subunits. The lack of concentration dependence for dissociation of large oligomers has been related to conformational drift of the dissociated monomers caused by substitution of intersubunit contacts by solvent–subunit contacts after dissociation (Ruan & Weber, 1989; Weber, 1992), which leads to microheterogeneity of conformations of the polymer upon reassembly. Given the small concentration dependence observed with vicilin (Figure 5), we have examined changes in the energetics of association and in conformation of the pressure-dissociated protein.

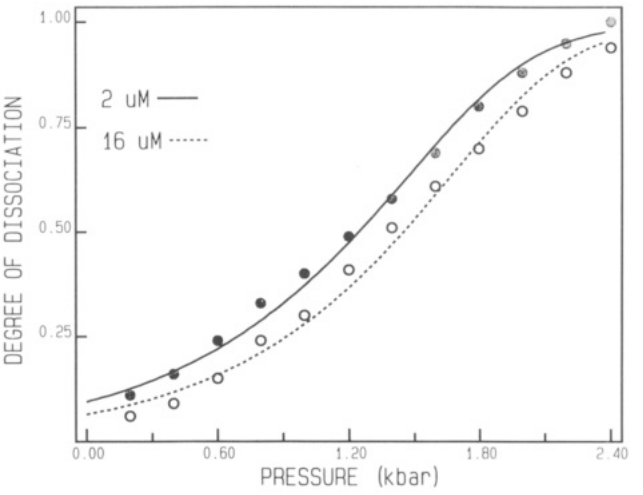


FIGURE 5: Concentration dependence for pressure dissociation of vicilin. Pressure dissociation was measured at pH 10 by following the decrease in fluorescence emission at 305 nm. Degrees of dissociation were calculated as described in the legend to Figure 3. Experiments were carried out using 2 (●) or 16 μM (○) vicilin. Symbols represent averages of eight experiments with standard deviations ranging from 0.01 to 0.11. Lines represent theoretical curves for pressure dissociation of a trimer fit to the data according to eq 3, as described under Materials and Methods.

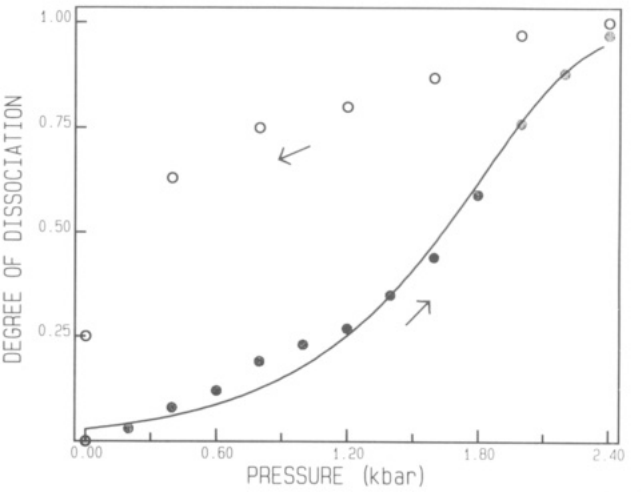


FIGURE 6: Compression–decompression cycle of vicilin. Vicilin (3.3 μM, pH 10) was compressed in 200 bar steps (10-min equilibration time at each pressure), and degrees of dissociation were calculated as in the legend to Figure 3 (●). After reaching 2.4 kbar, the sample was decompressed in 400 bar steps (10-min equilibration at each pressure) (○). Arrows indicate the directions of compression and decompression.

Figure 6 shows a compression-decompression cycle for vicilin. A pronounced hysteresis was observed in this cycle, indicating a significant loss of free energy of association of the subunits. The decrease in free energy of association ($d\Delta G$) can be calculated as

$$d\Delta G = -dp_{1/2}\Delta V^o \qquad (8)$$

From the data of Figure 6, a $d\Delta G$ value of approximately +5.0 kcal/mol was obtained.

The significant loss in free energy of association observed in the reassembly of pressure-dissociated vicilin indicated that conformational changes in the dissociated subunits decreased intersubunit affinity. We have thus examined changes in structure of the protein following pressure-dissociation. Figure 7 shows far-UV CD spectra of native vicilin, as well as of samples pressurized at 2.4 kbar for 10 or 60 min followed by decompression. Control measurements of the kinetics of

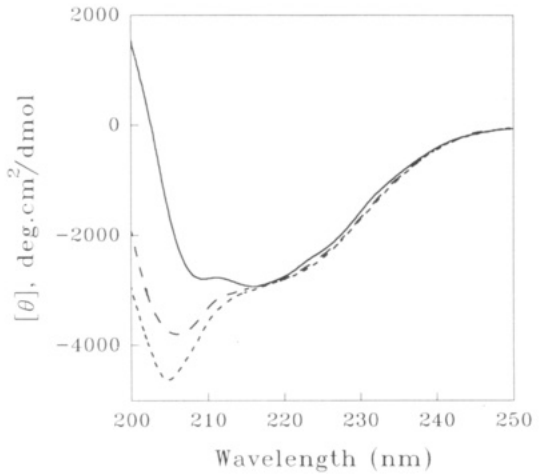


FIGURE 7: UV-CD spectra of native and pressurized vicilin. The UV-CD spectrum of nonpressurized vicilin (2.3 μM, pH 10) was measured as described under Materials and Methods (—). Samples were pressurized at 2.4 kbar for 10 min. (- - -) or 60 min. (···); pressure was then released, and CD spectra were measured at atmospheric pressure. No changes in CD spectra of pressurized samples occurred up to 3 h after pressure release.

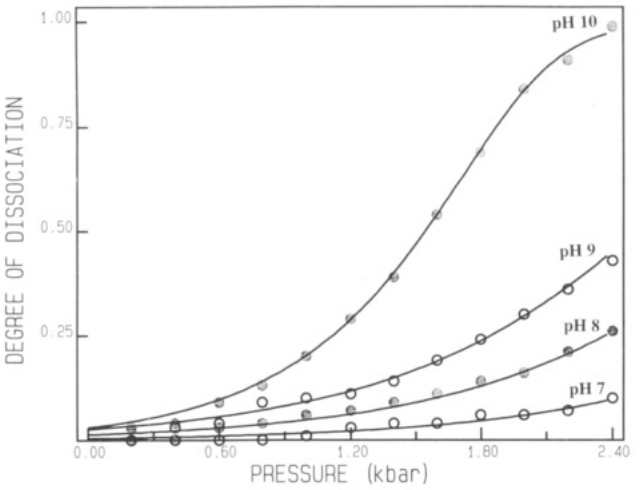


FIGURE 8: Effect of pH on pressure dissociation. Pressure dissociation of vicilin (3.3 μM) was carried out at pH 7, 8, 9, or pH 10, as indicated in the figure. Degrees of dissociation were calculated as described in the legend to Figure 3.

dissociation under pressure (not shown) indicated that 10 min was sufficient time for equilibration of pressure-dissociation of vicilin at each pressure. The far-UV CD spectrum of vicilin (measured at atmospheric pressure) after the protein was kept for 10 min at 2.4 kbar showed significant differences in the region between 200 and 215 nm relative to nonpressurized vicilin, indicating changes in secondary structure of the protein. The magnitude of the CD spectral changes was larger in samples which had been pressurized for 60 min, indicating slow time-dependent changes in the pressure-dissociated protein.

Destabilization of the Vicilin Oligomer at Alkaline pH. As shown in Figure 1, pressure-dissociation of vicilin was pH dependent. In order to characterize the pH-dependence for dissociation, we have carried out pressure–dissociation curves at different pH values from 7 to 10 (Figure 8). Alkalinization of the medium resulted in an increase in dissociation of vicilin. A sharp increase in pressure dissociation was observed when the pH was raised from 9 to 10 (Figure 8). The loss in free energy of subunit association caused by raising pH from 9 to 10 was calculated according to eq 8, and a $d\Delta G$ of +3.4 kcal/mol was found.

The effect of pH on dissociation by pressure was not related to denaturation of vicilin, since no significant changes were observed in the far-UV CD spectrum of vicilin at atmospheric pressure at pH 10 as compared to the spectrum at pH 8 (Figure 9A). Thus, the secondary structure of vicilin was unaffected by alkalinization to pH 10. Furthermore, measurements of the intrinsic fluorescence lifetimes of vicilin were used to probe the conformation of the protein at different pH values. Fluorescence lifetime data were analyzed with different models, as indicated under Materials and Methods. The fluorescence decay was found to be heterogeneous and was best described with a Lorentzian distribution of lifetime values[2] (Figure 9B). No significant changes in the fluorescence lifetime distribution occurred on changing pH from 8 to 10 (Figure 9B), indicating that the overall conformation of the protein was not significantly affected by alkalinization. Control measurements of the denaturation of vicilin with 6 M guanidine hydrochloride revealed 30% quenching of the average fluorescence lifetime (center of the lifetime distributions in Figure 9B) (not shown), indicating that the fluorescence decay measurements were sensitive to changes in the native conformation of vicilin.

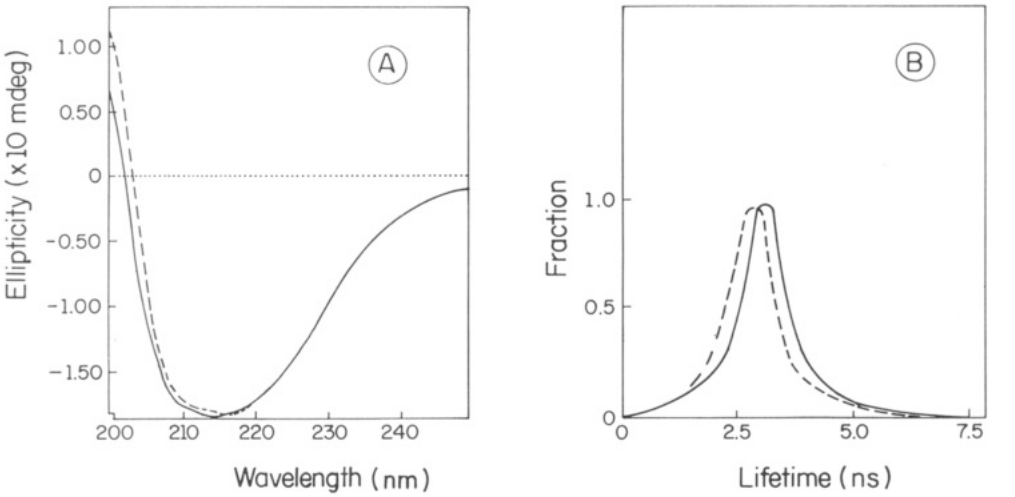
Stabilizing Effect of Cosolvents. Addition of 0.5 M salt promoted significant stabilization of the vicilin oligomer against pressure dissociation. Figure 10 shows pressure–dissociation curves in the presence of 0.5 M NaCl, KCl, or LiCl. All salts tested had comparable stabilizing effects. As seen in Figure 10, in the presence of 0.5 M salt it was not possible to reach half-maximal dissociation even at the highest pressure employed in this study (2.4 kbar). An estimate of the stabilization free energy involved could be obtained by fitting dissociation data at each pressure according to eq 3, as described under Materials and Methods. Free energies of dissociation were calculated from dissociation constants obtained from such fits, and the stabilization free-energy of association upon addition of 0.5 M salt was estimated to be about −1.2 to −2.4 kcal/mol.

Addition of glycerol promoted a dramatic stabilization of the oligomeric structure against pressure dissociation (Figure 11). The stabilizing effect of glycerol was further confirmed by FPLC gel filtration analysis of samples pressurized in the presence of 15% glycerol. Figure 12 shows gel filtration elution profiles of native vicilin (panel A) and of a sample which had been subjected to 2.4 kbar for 1 h in the presence of 15% glycerol (panel B). Glycerol promoted a significant stabilization of the 150-kDa vicilin oligomer, with a decrease in the relative amounts of smaller molecular weight components in the elution profile after pressure treatment (compare these results with the elution profiles obtained under identical conditions in the absence of glycerol, shown in Figure 2E and F).

DISCUSSION

In this work, perturbation of the equilibrium of subunit association by hydrostatic pressure has been used to provide insight into the mechanisms and energetics of subunit assembly in vicilin. Pressure-dissociation enabled determination of the standard molar volume change for association of subunits and the dissociation constant at atmospheric pressure. The very high affinity of intersubunit interactions in vicilin ($C_{1/2}$ = 18 nM) precluded direct determination of the equilibrium constant by more conventional techniques, such as dilution, and illustrates the usefulness of the methodology of pressure perturbation. Information regarding the nature of the

[2] S. T. Ferreira and C. Pedrosa, manuscript in preparation.



184q

FIGURE 9: Lack of effect of pH on vicilin structure at atmospheric pressure. (Panel A) UV-CD spectra of vicilin (2.3 μM) at atmospheric pressure at pH 8 (—) or pH 10 (- - -). (Panel B) Intrinsic fluorescence lifetime data were acquired and analyzed as described under Materials and Methods. The figure shows the Lorentzian fluorescence lifetime distributions obtained at pH 8 (—) or pH 10 (- - -). At pH 8, the distribution parameters were center = 3.1 ns, width = 1.0 ns, and χ^2 = 4.3. At pH 10, the parameters recovered were center = 2.9 ns, width = 1.0 ns, and χ^2 = 2.5.

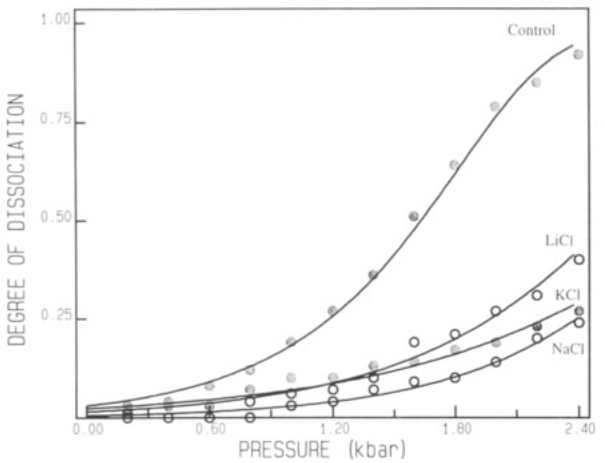


FIGURE 10: Effect of salts on pressure dissociation. Pressure dissociation of vicilin (3.3 μM, pH 10) was carried out in the absence of any added salts or in the presence of 0.5 M of LiCl, KCl, or NaCl, as indicated in the figure.

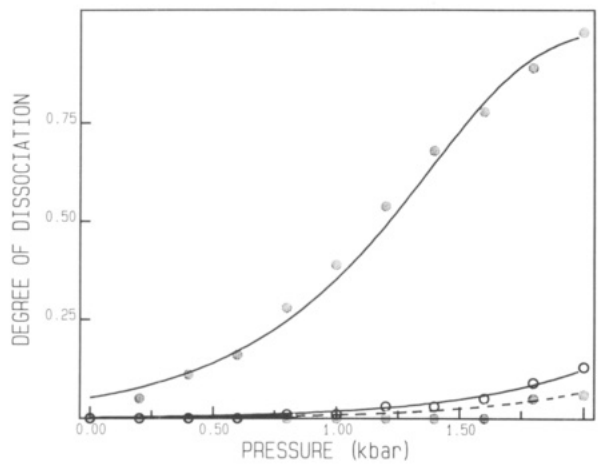


FIGURE 11: Effect of glycerol on pressure dissociation. Pressure dissociation of vicilin (3.3 μM, pH 10) was carried out in the absence of glycerol (●), or in the presence of 5% (○) or 15% (●, dashed line) glycerol.

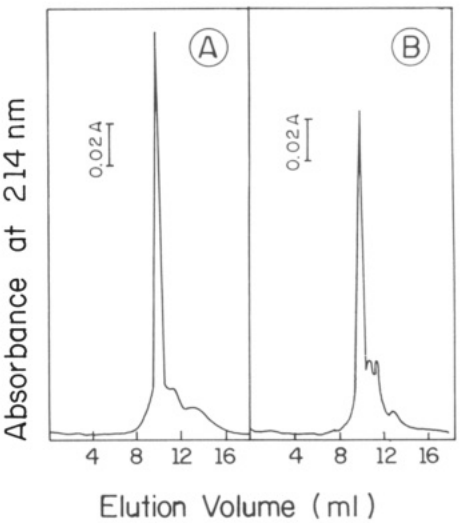


FIGURE 12: FPLC analysis of vicilin pressurized in the presence of glycerol. (Panel A) Size-exclusion chromatography profile of control, nonpressurized, vicilin sample (pH 10, containing 15% glycerol). (Panel B) A sample identical to that in panel A was pressurized at 2.4 kbar for 1 h, pressure was released, and an aliquot was immediately applied onto the FPLC column. Total protein applied in each run was 6 μg. Chromatography conditions are described under Materials and Methods.

interactions responsible for the stability of the vicilin oligomer could be gained from examination of the volume change of association of subunits. The volume change of association of vicilin (146 mL/mol) may be normalized to the molecular weight of the oligomer (Silva & Weber, 1993), thus furnishing a specific volume change of 1.0 μL/g. This is in the same range as specific volume changes of 0.7, 1.3, and 1.9 μL/g reported for enolase, hexokinase, and tryptophan synthase, respectively (Paladini & Weber, 1981; Silva et al., 1986; Ruan & Weber, 1988; Silva & Weber, 1993). Large values of volume change can be explained by hydration of charged groups previously involved in salt bridges between subunits (electrostriction; Newman et al., 1973). In contrast, for pressure dissociation of a detergent-solubilized membrane protein (the calcium-pump from erythrocyte plasma membrane) a specific volume change of 0.1–0.2 μL/g has been reported (Coelho-Sampaio et al., 1991), suggesting the participation of hydrophobic interactions in subunit association. The relatively high specific volume change observed for vicilin indicated likely participation of salt bridges in intersubunit interactions, a prediction which was supported by our pH studies (see below).

Deterministic Dissociation of Vicilin and Incomplete Reassembly of Dissociated Subunits. As expected from mass

action law, pressure dissociation should depend on protein concentration (Ruan & Weber, 1989). Such stochastic behavior has been observed in the pressure-dissociation of dimers, whereas large deviations from the predicted dependence on protein concentration for dissociation have been found in studies with larger protein aggregates [for reviews, see Weber (1992) and Silva and Weber (1993)]. The explanation that has been proposed to explain the low dependence (or independence) on protein concentration is that a solution of large protein aggregates constitutes a heterogeneous population, each member having its own characteristic dissociation pressure, resulting in a behavior similar to the deterministic equilibria of macroscopic bodies (Erijman & Weber, 1991). The transition from dimers to tetramers was reported to be sufficient to elicit deterministic behavior (King & Weber, 1986a,b; Ruan & Weber, 1989; Erijman & Weber, 1991). Here we found a lower protein concentration dependence for pressure-dissociation than predicted for vicilin, the observed $dp_{1/2}$ being about one-fourth of the expected value (Figure 5). In the only other case of a trimer which has been investigated (Foguel and Weber, personal communication), the dependence on protein concentration for dissociation was also lower than predicted. It is interesting to note that for tetrameric lactate dehydrogenase or glyceraldehyde-phosphate dehydrogenase the observed $dp_{1/2}$ were one-fourth to one-fifth of the predicted values (King & Weber, 1986a,b; Ruan & Weber, 1989). Thus, the deterministic pressure dissociation of vicilin is similar to that displayed by tetramers.

One possible origin of energetic heterogeneity of the oligomers has been proposed to be a conformational drift of the dissociated subunits (Weber, 1986, 1987). Conformational changes following dissociation have been found in many oligomeric proteins, regardless of the nature of the perturbation that promotes dissociation (Xu & Weber, 1982; King et al., 1986a,b; Silva et al., 1986, 1989; Ruan & Weber, 1988, 1989; Muga et al., 1990). Reassembly of modified subunits leads to heterogeneity in the oligomer population. These conformational changes often result in loss of free energy of subunit association, revealed by hysteresis in a compression–decompression cycle. A pronounced hysteresis was observed in the reassembly of vicilin from pressure-dissociated subunits (Figure 6), corresponding to a loss of 5 kcal/mol in stabilization free energy (i.e., about 25% of the total stabilization free energy of vicilin). The free energy loss probably arises from incorrect packing of conformationally drifted subunits. In fact, secondary structure changes in vicilin were evident after pressure-dissociation (Figure 7). It should be noted that the conformational changes undergone by the dissociated subunits occur in a slow time scale (minutes to hours, as revealed by the time dependence of CD spectral changes shown in Figure 7). This implies that the accumulation of conformationally "incorrect" subunits (i.e., those which will not reassemble correctly to the native oligomer) is a slow process compared to the kinetics of dissociation (which is complete in a few minutes).

Vicilin is synthesized as a trimer of intact 50-kDa subunits. The trimer then undergoes posttranslational processing (Gatehouse et al., 1982, 1983; Lycett et al., 1983) including proteolysis of some of the 50-kDa subunits and glycosylation. It is possible that the extent of posttranslational modification may vary among molecules, resulting in a heterogeneous population of oligomers containing different numbers of proteolytic nicks and relative amounts of carbohydrate attached. In addition, the expression of vicilin appears to be controlled by a family of genes (Croy et al., 1982; Lycett et al., 1983; Domoney & Casey, 1985), so that different isoforms of the 50-kDa subunit may actually be present. These may be important factors in determining the energetic heterogeneity of vicilin oligomers revealed by the low concentration dependence for dissociation.

The conformational changes that occur at degrees of dissociation close to unity explain the partial irreversibility of the dissociation of vicilin. Upon release of pressure after 1 h at 2.4 kbar (i.e., at high degree of dissociation) the fluorescence emission shows only partial reversibility to the native state (Figure 1), and an intermediate state of assembly (seen as a 75-kDa peak in the FPLC elution profiles in Figure 2) is detected. The FPLC profiles in Figure 2 were unmodified for 24 h following pressure release, indicating that the 75-kDa peak was a stable trapped intermediate in the assembly of the oligomer. The 75-kDa species probably consists of one 50-kDa subunit associated to one or more of the smaller peptides ranging from 12 to 33 kDa.

pH Effects on Pressure Dissociation. Pressure dissociation of vicilin was markedly increased at alkaline pH. Alkalinization did not affect the secondary structure or the overall conformation of vicilin at atmospheric pressure (Figure 9) but specifically rendered the quaternary association more sensitive to pressure perturbation. The sharp increase in pressure dissociation between pH 9 and 10 (Figure 8) suggests that deprotonation of side chains in this pH range (i.e., lysine residues) decreases the strength of intersubunit interactions. It appears likely, therefore, that lysine residues at subunit interfaces may participate in salt bridges which stabilize the oligomer.

It is interesting to compare the volume change observed for dissociation of vicilin with the volume change expected from disruption of a single salt bridge. In aqueous solvent, the volume change for the reaction

$$CH_3COOH + NH_3 \leftrightarrow CH_3COO^- + NH_4^+$$

is −17 mL/mol (Kauzmann et al., 1962). However, in a medium of lower dielectric constant (e.g., at intersubunit interfaces in proteins) this volume can be considerably more negative (Hamann, 1988). In the well-studied case of pressure destabilization of the salt bridge of chymotrypsin (Heremans & Wauters, 1980; Heremans & Heremans, 1989) volume changes of −30 to −35 mL/mol have been reported. As noted by Heremans and Heremans (1989), although it is not possible to separate the volume change for disruption of the salt bridge from that of conformational changes in the protein, for chymotrypsin most of the volume change appears related to the salt bridge. In a study of the role of coulombic interactions in the association between cytochrome b_5 and cytochrome c, Rodgers and Sligar (1991) showed that substitution of each charged residue at the interface led to a volume change of about −20 mL/mol. With these points in mind and considering the volume change of −146 mL/mol for dissociation of vicilin, one may calculate that disruption of 4–7 salt bridges could account for the observed volume change. The actual number of salt bridges may be smaller in view of the occurrence of conformational changes in vicilin subunits following dissociation (Figure 7) and of the possible existence of other types of interaction between subunits.

Upon deprotonation of a lysine residue originally participating in a salt bridge, the disrupted electrostatic bond might be replaced by a hydrogen bond between the neutral amino group and the carboxylate residue. This should lead to a decrease in volume change for subunit dissociation at high pH, since the volume change involved in disrupting a hydrogen bond is smaller than for an electrostatic bond. This was not observed with vicilin, since pressure dissociation curves in Figure 8 were fitted with the same ΔV^0 at different pH values.

Effects of pH on the association of seed storage proteins have previously been described. Grant and Lawrence (1964) showed that alkaline pH favored dissociation of pea vicilin. Legumin (the 11S storage protein) from various seeds was also shown to dissociate at extreme pH (>11) (Schwenke & Schultzmand, 1975; Prakash & Nandi, 1977; Pichl & Stokrova', 1982; Gueguen et al., 1988), as well as the 7S globulin from soybean (Koshiyama, 1971). However, at the high pH required for dissociation in these studies, denaturation of the proteins occurred concomitantly with their dissociation, thus complicating the interpretation of the results in terms of the effects on the oligomeric structure alone. One advantage of the methodology of pressure perturbation employed in the present study is that dissociation of vicilin could be investigated under more mild conditions (pH 10) which did not directly affect the secondary structure or the conformation of the protein.

Effects of Cosolvents. Pressure-dissociation of vicilin was significantly inhibited by addition of salts (Figure 10). The similarity of the results obtained with NaCl, KCl, or LiCl suggests that the effects are not due to specific interactions of a particular salt with the protein. The effect of salts may be related to an increase in surface tension of water and exclusion of the cosolvent from the solvation layer of the protein (Timasheff & Arakawa, 1989). This latter mechanism, involving preferential hydration of the protein in the presence of high concentrations of cosolvents, has been proposed to explain the stabilizing effects of various salts and/or organic solutes on protein structure [for reviews, see Timasheff and Arakawa (1989) and Timasheff (1993)]. Addition of salts may influence the organization of water around polar groups at the protein–water interface (Low & Somero, 1975), thus changing the chemical potential of the dissociated subunits so as to favor the oligomer. Alternatively, the stabilizing effect of salts might also be explained by general charge screening in the protein, which could result in a decrease in destabilizing repulsive interactions between subunits.

Qualitatively similar protective effects of salts on pressure dissociation of the P_2 isoenzyme of hexokinase (Ruan & Weber, 1988) and on *G. paulistus* erythrocruorin (Bonafe et al., 1991) have been reported. Salt effects on seed storage proteins have also been described. Schwenke and Schultzmand (1975) reported stabilization of oligomeric sunflower legumin by high ionic strength, while Gueguen et al. (1988) and Koshiyama (1971) showed similar effects on pea legumin and on the 7S globulin of soybean, respectively. Thus, maybe these previous observations with different proteins, as well as our results with pea vicilin, can all be explained in terms of the preferential hydration mechanism. In contrast, salts of the Hofmeister series have been shown to promote dissociation of pea legumin (Chambers et al., 1990), and at high concentrations of these salts dissociation is accompanied by partial denaturation.

Addition of glycerol to the medium also promoted a marked stabilization of oligomeric vicilin against pressure dissociation (Figures 11 and 12). Pressure dissociation was almost completely blocked at 15% (2.1 M) glycerol. The effect of glycerol may likely be explained by preferential hydration, as proposed by Timasheff and co-workers (Timasheff, 1993). In this regard, it is interesting to consider the number of water molecules bound to the dissociated interfaces. As pointed out by Silva and Weber (1993), this can be calculated from the measured volume change of dissociation assuming a cross section of 10 $Å^2$ for a water molecule, yielding a value of 48 water molecules for vicilin. Hydration of the cosolvents prevents water from binding at the subunit interfaces, thus stabilizing the oligomer. Stabilizing effects of glycerol on the oligomeric structures of tubulin microtubules (Lee & Timasheff, 1975, 1977), erythrocruorin from *G. paulistus* (Bonafe et al., 1991), and soluble F_1-ATPase (Dreyfus et al., 1988) have been reported. In all these cases, stabilization appears due to exclusion of glycerol from the dissociated subunit interfaces thus favoring the assembled form.

We have also considered the possibility that the effect of glycerol on the pressure dissociation of vicilin could be due to changes in viscosity, dielectric constant or pH of the solutions upon addition of 5–15% (v/v) glycerol. However, we note that at 20 °C addition of 5% or 15% glycerol produces only modest increases in viscosity (from 1.005 cp in the absence of glycerol to 1.143 cp or 1.517 cp with 5% or 15% glycerol, respectively; Miner & Dalton, 1953). In addition, the dielectric constant of glycerol–water mixtures changes only from 80.4 in the absence of glycerol to 79 in the presence of 5% glycerol or to 76.2 in the presence of 15% glycerol (Miner & Dalton, 1953). Since pH was an important variable in our studies of the pressure dissociation of vicilin, we have routinely checked the pH of our vicilin solutions, including those containing glycerol. Furthermore, glycerol is a very weak acid (with a dissociation constant of 7×10^{-15} M; Miner & Dalton, 1953), and therefore it is not expected to produce significant changes in pH of buffered solutions. These considerations suggest that the marked protective effects of glycerol against pressure dissociation of vicilin are not likely explained by changes in viscosity, dielectric constant, or pH of the solutions. In any case, the protection conferred by glycerol should be qualitatively viewed as an effect of cosolvent (Timasheff, 1993), and further studies employing different cosolvent systems might enable more quantitative correlations to be made.

Possible Physiological Roles of Solutes in the Assembly of Vicilin. Bonafe et al. (1991) suggested that the effects of nonprotein factors such as different cosolvents may be physiologically relevant to the assembly of oligomeric proteins *in vivo*. Cosolvents could act to promote correct assembly of subunits and to prevent accumulation of wrong intermediates due to conformational drift of isolated polypeptides (Bonafe et al., 1991). Seed storage proteins are synthesized in the rough endoplasmic reticulum and inserted into the endoplasmic reticulum for transport to the protein bodies of the seeds. Both in the endoplasmic reticulum and in the protein bodies the storage proteins may be exposed to high salt concentrations which might contribute to their correct assembly. As discussed above, the stabilizing effect of glycerol may involve changes in organization or activity of water around the protein. Therefore, while glycerol itself possibly does not play a role under physiological conditions, its effect may mimic what happens during the process of dehydration of the seed. Similar effects could be exerted *in vivo* by other polyols such as the carbohydrates stored in the seeds. It is thus tempting to speculate that in the seeds different solutes could act to promote the correct formation of tertiary or quaternary structures of proteins. In this regard, these could be auxiliary mechanisms to the action of the chaperonins (Ellis & Hemmingsen, 1989) which have been identified to participate in the folding or assembly of plant storage proteins (Gething & Sambrook, 1992).

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REFERENCES

Baniel, A., Caer, D., Colas, B., & Gueguen, J. (1992) *J. Agric. Food Chem. 40*, 200–205.
Beechem, J. M., Gratton, E., Ameloot, M., Knutson, J. R., & Brand, L. (1991) In *Topics in Fluorescence Spectroscopy, Vol. 2, Principles* (Lakowicz, J. R., Ed.) pp 241–305, Plenum Press, New York.
Bewley, J. D., & Black, M. (1985) *Seeds Physiology, Development and Germination*, Plenum Press, New York.
Bonafe, C. F. S., Villas-Boas, M., Suarez, M. C., & Silva, J. L. (1991) *J. Biol. Chem. 266*, 13210–13216.
Brand, B. P., & Johnson, P. (1956) *Trans. Faraday Soc. 52*, 438–451.
Caer, D., Baniel, A., Subirade, M., Gueguen, J., & Colas, B. (1990) *J. Agric. Food Chem. 38*, 1700–1706.
Chambers, S. J., Carr, H. J., & Lambert, N. (1990) *Biochim. Biophys. Acta 1037*, 66–72.
Chandra, B. R., Prakash, V., & Rao, M. S. N. (1985) *J. Biosci. 9*, 177–184.
Coelho-Sampaio, T., Ferreira, S. T., Benaim, G., & Vieyra, A. (1991) *J. Biol. Chem. 266*, 22266–22272.
Croy, R. R. D., Lycett, G. W., Gatehouse, J. A., Yarwood, J. N., & Boulter, D. (1982) *Nature 295*, 76–79.
Domoney, C., & Casey, R. (1985) *Nucleic Acids Res. 13*, 687–699.
Dreyfus, G., Guimaraes-Motta, H., & Silva, J. L. (1988) *Biochemistry 27*, 6704–6710.
Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends. Biochem. Sci. 14*, 339–342.
Erijman, L., & Weber, G. (1991) *Biochemistry 30*, 1595–1599.
Ferreira, S. T., & Verjovski-Almeida, S. (1989) *J. Biol. Chem. 264*, 15392–15397.
Ferreira, S. T., & Verjovski-Almeida, S. (1991) *J. Lumin. 48*, 430–434.
Garcia, C. R., Amaral, Jr., A. J., Abrahamsohn, P., & Verjovski-Almeida, S. (1992) *Eur. J. Biochem. 209*, 1005–1011.
Gatehouse, J. A., Lycett, G. W., Croy, R. R. D., & Boulter, D. (1982) *Biochem. J. 207*, 629–632.
Gatehouse, J. A., Lycett, G. W., Delauney, A. J., Croy, R. R. D., & Boulter, D. (1983) *Biochem. J. 212*, 427–432.
Gatehouse, J. A., Croy, R. R. D., & Boulter, D. (1984) *CRC Crit. Rev. Plant Sci. I (4)*, 287–314.
Gething, M. J., & Sambrook, J. (1992) *Nature 355*, 33–45.
Gueguen, J., Chevalier, M., Barbot, J., & Schaeffer, F. (1988) *J. Sci. Food Agric. 44*, 167–182.
Guerrieri, N., & Cerletti, P. (1990) *J. Protein Chem. 9*, 397–405.
Grant, D. R., & Lawrence, J. M. (1964) *Arch. Biochem. Biophys. 108*, 552–561.
Hamann, S. D. (1988) in *Organic High Pressure Chemistry* (Le Noble, W. J., Ed.) pp 50–99, Elsevier, Amsterdam.
Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.
Heremans, K., & Wauters, J. (1980) in *High Pressure Science and Technology* (Vodar, B., & Marteau, P., Eds.) pp 845–847, Pergamon Press, New York.
Heremans, L., & Heremans, K. (1989) *Biochim. Biophys. Acta 999*, 192–197.
Kauzmann, W., Bodanszky, A., & Rasper, J. (1962) *J. Am. Chem. Soc. 84*, 1777–1788.
King, L., & Weber, G. (1986a) *Biochemistry 25*, 3632–3637.
King, L., & Weber, G. (1986b) *Biochemistry 25*, 3637–3640.
Kinsella, J. E. (1979) *J. Am. Oil Chem. Soc. 56*, 242–258.
Koshiyama, I. (1971) *Agric. Biol. Chem. 35*, 385–392.
Lambert, N., Chambers, S. J., Phalp, M., & Wright, D. J. (1986) *Biochem. Soc. Trans. 14*, 1186–1188.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.
Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.
Low, P. S., & Somero, G. N. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3305.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Kandall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lycett, G. W., Delauney, A. J., Gatehouse, J. A., Croy, R. R. D., & Boulter, D. (1983) *Nucleic Acids Res. 11*, 2367–2380.
Miner, C. S., & Dalton, N. N. (1953) *Glycerol*, Reinhold Publishing Corp., New York.
Muga, A., Surewicz, W. K., Wong, P. T., Mantsch, H. H., Singh, V. K., & Shinohara, T. (1990) *Biochemistry 29*, 2925–2930.
Newman, R. C., Kauzmann, W., & Zipp, J. (1973) *J. Phys. Chem. 77*, 2687–2691.
Paladini, A., Jr., & Weber, G. (1981) *Biochemistry 20*, 2587–2593.
Pichl, I., & Stokrová, S. (1982) *Biochem. Physiol. Pflanz. 177*, 547–555.
Prakash, V., & Nandi, P. K. (1977) *Int. J. Pept. Protein Res. 9*, 319–328.
Rodgers, K. K., & Sligar, S. G. (1991) *J. Mol. Biol. 221*, 1453–1460.
Ruan, K., & Weber, G. (1988) *Biochemistry 27*, 3295–3301.
Ruan, K., & Weber, G. (1989) *Biochemistry 28*, 2144–2153.
Schwenke, K. D., & Schultzmand, L. K. J. (1975) *Die Nabrung 19*, 425–432.
Silva, J. L., & Weber, G. (1988) *J. Mol. Biol. 199*, 149–159.
Silva, J. L., & Weber, G. (1994) *Annu. Rev. Phys. Chem. 44*, (in press).
Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5780–5786.
Silva, J. L., Villas-Boas, M., Bonafé, C. F. S., & Meirelles, N. (1989) *J. Biol. Chem. 264*, 15863–15868.
Thompson, R. B., & Lakowicz, J. R. (1984) *Biochemistry 23*, 3411–3417.
Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67–97.
Timasheff, S. N., & Arakawa, T. (1989) in *Protein Structure, A Practical Approach* (Creighton, T. E., Ed.) pp 331–345, IRL Press, Oxford.
Verjovski-Almeida, S., Kurtenbach, E., Amorim, A. F., & Weber, G. (1986) *J. Biol. Chem. 261*, 9872–9878.
Watson, M. D., Lambert, N., Delauney, A. J., Yarwood, J. F., Croy, R. R. D., Gatehouse, J. A., Wright, D. J., & Boulter, D. (1988) *Biochem. J. 251*, 857–864.
Weber, G. (1986) *Biochemistry 25*, 3626–3631.
Weber, G. (1987) in High Pressure Chemistry and Biochemistry 197, *NATO ASI Ser., Ser. C 197*, 401–420.
Weber, G. (1992) *Protein Interactions*, Chapman and Hall, New York.
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 116*, 89–112.
Wright, D. J. (1985) in *Developments in Food Proteins*, Vol. 5, pp 81–157, Elsevier Applied Science, London.
Wright, D. J., & Bumstead, M. R. (1984) *Phil. Trans. R. Soc. Lond. B 304*, 381–393.
Xu, G.-J., & Weber, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5268–5271.